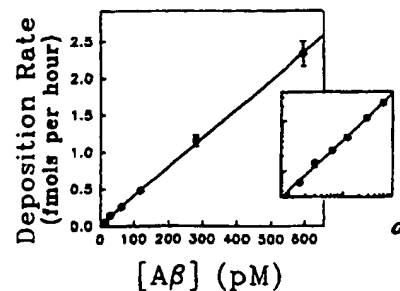
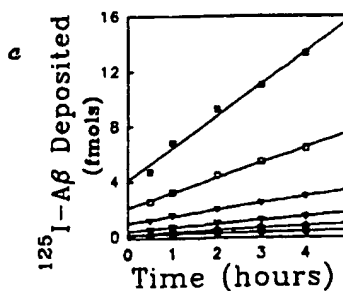
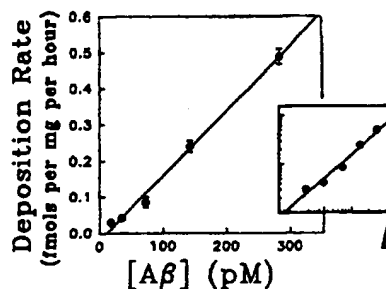
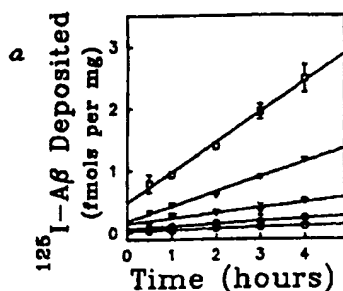




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(54) Title: A $\beta$  DEPOSITION INHIBITOR SCREEN USING SYNTHETIC AMYLOID

## (57) Abstract

The present invention provides synthetic amyloid, i.e., a synthetic analog of naturally occurring amyloid, which includes a synthetic aggregate of one or more synthetic or naturally occurring peptides. Preferably, the aggregate possesses a fibrillar morphology. This synthetic material can serve as a template for deposition of A $\beta$  peptide and/or active fragments/modifications thereof.

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## A $\beta$ Deposition Inhibitor Screen Using Synthetic Amyloid

### Background of the Invention

5 Alzheimer's disease (AD) is a progressive senile dementia affecting a significant fraction of the elderly. The defining pathological feature of the disease is the presence of numerous insoluble deposits described as amyloid on the basis of tinctorial properties, in the brains of affected patients. The major constituent of AD amyloid deposits is a hydrophobic 35- to 43-amino  
10 acid peptide termed the  $\beta$ -amyloid peptide (A $\beta$ ), which is produced as a cleavage product of a much larger transmembrane protein, the amyloid precursor protein ( $\beta$ PP) encoded by the amyloid precursor protein gene (*APP*).

It is becoming increasingly clear that A $\beta$  deposition contributes directly to the progressive neurodegeneration in AD. Strong circumstantial  
15 evidence that A $\beta$  plays a causative rather than consequential role in Alzheimer's disease has come from several lines of research. The A $\beta$  peptide is toxic to neurons in culture, but only in a fibrillar (amyloid) state, and A $\beta$  overproduction in the brains of transgenic mice leads to both significant A $\beta$  deposition and neuronal toxicity. A $\beta$  deposition is essentially an invariant feature of AD and in  
20 careful studies, there is a strong correlation between amyloid burden at death and the degree of dementia during life. Furthermore, several genetically heritable forms of AD are tightly linked to mutations in A $\beta$  or *APP*. Other AD genetic loci not linked to *APP* have been shown to affect  $\beta$ PP (amyloid precursor protein) processing to A $\beta$ .

25 A $\beta$  is a natural product rather than a pathological one. The peptide is found at similar concentrations (approximately nM concentrations) in normal and AD plasma and cerebrospinal fluid (CSF) and is constitutively produced by cells in culture. A $\beta$  has low solubility and at high concentrations ( $\mu$ M to mM) assembles spontaneously into fibrils that are morphologically  
30 similar to those found in AD amyloid by several criteria.

Attempts to target amyloid formation and deposition for therapeutic intervention in AD have followed diverse strategies. Because A $\beta$  is

produced by proteolysis of  $\beta$ PP, specific inhibition of the enzymes responsible for its production is an attractive target, but one that presents some obstacles. The proteases ( $\beta$ - and  $\gamma$ -secretase) responsible for  $A\beta$  production have not been identified and a nonpathogenic role for these enzymes has not been ruled out.

- 5    Affecting  $\beta$ PP metabolism through pharmacological intervention may also have detrimental consequence as a "normal" role for  $\beta$ PP or its fragments has not been excluded; *APP* null mice do display an abnormal phenotype.

A second common approach is the identification of compounds that block  $A\beta$  aggregation (the tendency of a molecule or colloidal body to  
10    associate together into a mass or body of units or parts) *in vitro*, which may be accomplished by high-throughput screening. However, spontaneous aggregation of  $A\beta$  *in vitro* does not occur at physiological concentrations, and aggregation assays are thus performed at  $A\beta$  concentrations several orders of magnitude higher than those found in CSF. The mechanism by which the peptide  
15    assembles may be quite different at physiological concentrations *in vivo*. Furthermore, the endpoint of aggregation assays almost surely represents a multi-step process, making the precise mechanism of action of an inhibitor difficult to determine. Although  $A\beta$  nucleation and aggregation may be an initial step in amyloid template formation, recent work has demonstrated that the  
20    process of  $A\beta$  deposition onto a pre-existing amyloid template is independent of nucleation and aggregation. Although inhibitors of  $A\beta$  nucleation might be useful prophylactically to prevent initial template formation,  $A\beta$  deposition onto that template may or may not be affected by inhibitors identified in aggregation screens.

- 25             $A\beta$  deposition can be modeled *in vitro* under near physiological conditions by monitoring the deposition of synthetic radiolabeled  $A\beta$  at physiological concentration onto AD amyloid in unfixed preparations (slide-mounted sections or homogenates) of AD human cerebral cortex. This model system has been used to elucidate key mechanistic and conformational  
30    features of  $A\beta$  for deposition, and works well for characterization of agents which affect deposition rates. See, for example, J.E. Maggio et al., Brain Pathol., 6, 147-162 (1996); W.P. Esler et al., Biochemistry, 35, 749-757 (1996);

P.W. Mantyh et al., Bull. Clin. Neurosci., 56, 73-85 (1991); J.E. Maggio et al. Proc. Natl. Acad. Sci. USA, 89, 5462-5466 (1992); J.P. Lee et al., Biochemistry, 34, 5191-5200 (1995); W.P. Esler et al., Biochemistry, 44, 13914-13921 (1996); and U.S. Patent No. 5,434,050 (Maggio et al.). The requirements for human  
5 tissue and centrifugation, however, make this assay cumbersome for high throughput screening.

What is needed is a system that is amenable to high throughput screening for the identification of A $\beta$  deposition inhibitors under physiological conditions. Specifically, what is needed is a template for A $\beta$  deposition  
10 (including A $\beta$  peptide or active fragments thereof) that closely resembles A $\beta$  deposition onto plaques in AD brain tissue. One such system involves the use of silk, such as spider silk or silkworm silk, as a template for A $\beta$  deposition, as disclosed in Applicants' Assignees' U.S. Patent Application Serial No. 08/304,585 filed September 12, 1994; however, additional materials and  
15 methods are still desired.

### Summary of the Invention

The present invention provides synthetic amyloid, i.e., a synthetic analog of naturally occurring amyloid, which includes a synthetic aggregate of  
20 one or more synthetic or naturally occurring peptides. Preferably, the aggregate possess a fibrillar morphology. This synthetic material can serve as a template for deposition of A $\beta$  peptide and/or active fragments/modifications thereof. Significantly, this deposition closely resembles A $\beta$  deposition onto plaques in AD brain tissue. This is surprising because there are millions of components in  
25 AD brain tissue and dozens of components in AD plaques that would have been expected to be needed to achieve a workable model.

In particularly preferred embodiments, the synthetic amyloid is immobilized. Immobilized synthetic amyloid is amenable to high throughput screening for the identification of A $\beta$  deposition inhibitors under physiological  
30 conditions. Thus, the present invention also provides a method for identifying A $\beta$  deposition inhibitors.

Specifically, the present invention provides an immobilized

synthetic amyloid comprising one or more peptides aggregated to form a peptide aggregate in a fibrillar morphology, wherein the peptides of the peptide aggregate are chosen such that a  $\beta$ -amyloid peptide, an active fragment thereof, or an active modification of  $\beta$ -amyloid peptide or fragment thereof binds to the peptide aggregate. Preferably, the synthetic amyloid comprises one or more aggregated peptides immobilized in an organic polymeric matrix. Preferably, the one or more aggregated peptides comprises a  $\beta$ -amyloid peptide, an active fragment thereof, or an active modification of  $\beta$ -amyloid peptide or a fragment thereof.

The present invention also provides a method of making immobilized synthetic amyloid, the method comprising: aggregating one or more peptides under conditions effective to form a fibrillar peptide aggregate; wherein the peptides of the peptide aggregate are chosen such that a  $\beta$ -amyloid peptide, an active fragment thereof, or an active modification of  $\beta$ -amyloid peptide or fragment thereof binds to the peptide aggregate; and combining the fibrillar aggregate with an organic polymer to form an immobilized synthetic amyloid. Preferably, the step of aggregating one or more peptides comprises: dissolving one or more peptides in an aqueous medium having a pH of about 6.5 to about 7.5 to form an aqueous peptide solution; and agitating the aqueous peptide solution under conditions effective to form a fibrillar peptide aggregate.

A particularly significant aspect of the present invention is an *in vitro* method of screening an agent capable of affecting A $\beta$  deposition, comprising: combining a sample of synthetic amyloid with an A $\beta$  tracer and a potential deposition-affecting agent to be screened, for a time effective to allow binding of the A $\beta$  tracer to the synthetic amyloid; wherein the synthetic amyloid comprises one or more aggregated peptides and the A $\beta$  tracer comprises a labelled  $\beta$ -amyloid peptide, active fragment thereof, or active modification of  $\beta$ -amyloid peptide or its fragments; detecting the amount of A $\beta$  tracer bound to the synthetic amyloid; and assessing the effect of the agent on the amount of A $\beta$  tracer bound to the synthetic amyloid. Although this method involves detecting an amount of bound tracer, this can be done either qualitatively or quantitatively.

The present invention also provides a multi-well plate comprising synthetic amyloid, wherein the synthetic amyloid comprises one or more peptides aggregated to form a peptide aggregate in a fibrillar morphology immobilized in an organic polymeric matrix, wherein the peptides of the peptide  
5 aggregate are chosen such that a  $\beta$ -amyloid peptide, an active fragment thereof, or an active modification of  $\beta$ -amyloid peptide or fragment thereof binds to the peptide aggregate.

The present invention also provides a method of identifying an active fragment or modification of  $\beta$ -amyloid peptide comprising: combining a  
10 sample of synthetic amyloid with an amount of labelled fragment or modification of  $\beta$ -amyloid peptide for a time effective to allow binding of the labelled fragment or modification to the synthetic amyloid, wherein the synthetic amyloid comprises one or more peptides aggregated to form a peptide aggregate; wherein the peptides of the peptide aggregate are chosen such that a  $\beta$ -amyloid  
15 peptide binds to the peptide aggregate; and detecting the presence of the labelled peptide fragment or modification bound to the synthetic amyloid.

As used herein, the term "synthetic" refers to materials that are not naturally occurring, although the constituent parts (e.g., the peptide and organic polymer) can be naturally occurring. Thus, the term "synthetic amyloid"  
20 refers to a material that functions as a model for amyloid that is not naturally occurring, thereby excluding naturally occurring amyloid in brain tissue as well as silk, which has been shown to be an acceptable model for amyloid, for example. The term "aggregate" refers to molecules or colloidal bodies associated into a mass or body of units or parts. The term "fibrillar" refers to the  
25 morphology of the aggregate, which is fibril- or fiber-like. The term "deposition" refers to the tendency of a material, e.g., a molecule or colloidal body, to associate with or adhere to a preexisting mass or body of units constituting a solid structure. In deposition, a material previously in solution deposits onto a preexisting structure, thus being removed from solution and  
30 forming a complex with the preexisting structure. The term A $\beta$  tracer includes labelled A $\beta$  peptide, labelled active fragments thereof, and/or labelled active modifications of A $\beta$  peptide and its fragments.

### **Brief Description of the Drawings**

**Figure 1:** Images (a-d) are darkfield autoradiograms with light areas indicating sites of radiolabeled  $^{125}\text{I}$ -A $\beta$  deposits onto amyloid in AD cortex (a) and onto the synthetic amyloid template of the present invention (c) but not onto comparable preparations of amyloid-free (control) cortex (b) or preparations of unaggregated A $\beta$  (d). Scale bar (panels a-d) = 4 mm. Images (e) and (f) are fluorescent micrographs of Thioflavin S stained synthetic amyloid template (e) and the comparable preparation of unaggregated A $\beta$  (f). Scale bar (panels e-f) = 0.6 mm.

**Figure 2:** Graphs (a) and (c) are time course deposition plots of the deposition of radiolabeled A $\beta$  onto AD cortex homogenates (a) or synthetic amyloid template (c). A $\beta$  at approximately 600 pM (■), 300 pM (□), 150 pM (▼), 75 pM (▽), 36 pM (●), and 18 pM (○). Graphs (b) and (d) are kinetic plots of A $\beta$  deposition rate onto AD cortex (b) or synthetic amyloid template (d) vs. concentration. Insets (b) and (d) are log-log plots of deposition rate vs. A $\beta$  concentration for AD cortex (b inset) or synthetic amyloid template (d inset).

**Figure 3:** (a) Graph of A $\beta$  deposition onto AD cortex (●) or synthetic amyloid template (○) measured at several pH values. (b) Graph of the rates of deposition of several A $\beta$  analogs and A $\beta$ (1-40) (defined as 100%) onto both AD cortex (abscissa) and synthetic amyloid template (ordinate). Peptides tested were A $\beta$ (1-40) (■), A $\beta$ (10-35)-NH<sub>2</sub> (□), A $\beta$ (10-35)-NH<sub>2</sub> L17T, F19T (●), A $\beta$ (10-35)-NH<sub>2</sub> F20I (▼), A $\beta$ (10-35)-NH<sub>2</sub> A21V (▲) and Y<sup>o</sup>-A $\beta$ (23-35) (○).

**Figure 4:** Dose response profiles for A $\beta$  deposition -- congo red (a) or urea (b) -- on synthetic amyloid template (○) or AD cortex (●). Bar chart (c) shows A $\beta$  deposition on synthetic amyloid template preincubated with 1 mM Congo red, 0.3 M urea, or buffer (50 mM TrisHCl, 0.1% BSA, pH 7.5). Darkfield autoradiograms (d-f) show sites of A $\beta$  deposition (light areas) after incubation of AD cortex sections with 100 pM radiolabeled A $\beta$  and 3 mM Congo red (d), buffer (50 mM TrisHCl, 0.1% BSA, pH 7.5) (e), or 1 M urea (f). Scale bar = 7 mm.



**Figure 5:** (a) Compounds examined for the ability to inhibit or accelerate A $\beta$  deposition onto synthetic amyloid template and authentic brain amyloid in preparations of AD cortex. (b) Graphical representation of the ability of these compounds to inhibit or accelerate A $\beta$  deposition onto synthetic amyloid template and authentic brain amyloid in preparations of AD cortex. (c) Graphical representation of the potency for inhibition or acceleration of A $\beta$  deposition onto AD cortex compared with the potency of inhibition or acceleration of aggregation estimated from published reports for rifampicin, ZnCl<sub>2</sub>, MTMA and Congo red.

10

#### **Detailed Description of Preferred Embodiments**

The formation, growth, and maturation of brain amyloid "senile" plaques are essential pathological processes in Alzheimer's disease and key targets for therapeutic intervention. The process of *in vitro* deposition of A $\beta$  peptide or fragments thereof at physiological concentrations onto plaques in AD brain preparations has been well characterized, but is cumbersome for drug discovery. Thus, preferred embodiments of the present invention provides a high throughput screening test for inhibitors of A $\beta$  deposition onto a synthetic amyloid template. This synthetic template is substantially indistinguishable from plaques in AD brain (the natural template) in deposition kinetics, pH profile, and structure-activity relationships for both A $\beta$  analogs and inhibitors. In contrast to conventional A $\beta$  aggregation screens, the synthetic template of the present invention accurately predicts inhibitor potency for A $\beta$  deposition onto AD cortex preparations (see Figure 5), validating its use in searching for agents that could slow the progression of AD and exposing a previously inaccessible target for drug discovery.

The present invention provides synthetic amyloid, which includes an aggregate of one or more synthetic or naturally occurring peptides. These peptides (i.e., sequences of a wide number of amino acids, typically, sequences of about 10 amino acids to several thousand amino acids, for example) are chosen such that  $\beta$ -amyloid peptide (i.e., A $\beta$  peptide), active peptide fragments thereof, or active modifications of  $\beta$ -amyloid peptide and its fragments bind to

the synthetic amyloid. In this context, the peptides that are used in the formation of the synthetic amyloid can be a wide variety of peptides as long as upon aggregation they bind to A $\beta$  amyloid peptide and/or fragments/modifications that are active for (i.e., bind to, as defined below) naturally occurring amyloid.

5 Thus, although the major constituent of naturally occurring amyloid deposits is A $\beta$  peptide, the synthetic amyloid of the present invention can include peptides other than A $\beta$  peptide as long as A $\beta$  peptide, active fragments, and/or active modifications of A $\beta$  peptide and its fragments bind to the synthetic amyloid. In this way, the synthetic amyloid functions as a template for A $\beta$  deposition, and

10 therefore, functions as a model for AD tissue.

Preferably, the aggregates are in a fibrillar morphology, analogous to that of naturally occurring amyloid. More preferably, the synthetic amyloid of the present invention includes an aggregate of A $\beta$  peptide, active fragments thereof, as well as active modifications of A $\beta$  peptide and its

15 fragments. Such peptides may be synthetic or naturally occurring.

Synthetic amyloid of the present invention can be used in methods for identifying A $\beta$  deposition inhibitors (e.g., methods for screening drugs that may be of therapeutic value in the treatment of amyloidoses). Typically, such methods involve contacting the synthetic amyloid with an A $\beta$

20 tracer. As used herein, an A $\beta$  tracer includes labelled A $\beta$  peptide, labelled active fragments thereof, and/or labelled active modifications of A $\beta$  peptide and its fragments. Synthetic amyloid of the present invention can also be used in methods for identifying active  $\beta$ -amyloid fragments or modifications or for studying the mechanism of A $\beta$  deposition.

25 For additional advantages in the methods of the present invention, particularly in drug screening tests, synthetic amyloid of the present invention is preferably immobilized. Suitable immobilization techniques are those that allow the peptide aggregate(s) to be available for binding to the A $\beta$  tracer (i.e., labelled A $\beta$  peptide or active fragments/modifications thereof). A wide variety of such

30 techniques are known to one of skill in the art. For example, aggregated peptide can adhere to the surface of a substrate, such as glass or plastic, by adsorption, hydrogen bonding, etc. Alternatively, aggregated peptide can be attached to a

substrate, such as beads or resins, using affinity binding techniques (e.g., antibody binding techniques). Also, aggregated peptide can be immobilized in a matrix of an organic polymer, which is preferred for the methods of the present invention.

5           As stated above, the synthetic amyloid of the present invention can include aggregated naturally occurring or synthetic peptides. Preferably, the peptides include synthetic or naturally occurring  $\beta$ -amyloid peptide, fragments (i.e., portions of A $\beta$  peptide) thereof, as well as modifications (e.g., which include amino acid replacements, substitutions, rearrangements, stereochemical  
10       modifications, etc.) of  $\beta$ -amyloid peptide and its fragments. Preferably, the peptides used in preparing the synthetic amyloid of the present invention include  $\beta$ -amyloid peptide, active fragments thereof, and active modifications of  $\beta$ -amyloid peptide and its fragments, wherein such peptides are "active" toward naturally occurring amyloid plaque derived from Alzheimer's disease tissue as  
15       defined below.

          In synthetic amyloid, whether immobilized or not, the peptide is in the form of an aggregate, preferably a fibrillary aggregate. Although aggregation can occur under a wide variety of conditions with respect to concentration, pH, salt content, buffer content, temperature, and the like, such  
20       conditions can vary depending on the peptide. Furthermore, aggregation may result in aggregates having varying degrees of effectiveness with respect to A $\beta$  deposition. Thus, the formation of effective synthetic amyloid templates may require optimizing the conditions for each particular peptide chosen.

          To a significant extent, aggregation of the peptide, particularly  $\beta$ -  
25       amyloid peptide, is dependent on the concentration of the peptide in solution prior to aggregation. For example, an about  $10^{-4}$  molar aqueous buffer solution of  $\beta$ -amyloid peptide<sup>1-40</sup> will commence self-aggregation at room temperature (about 20°C to about 25°C). Aggregation is also sensitive to the pH and salt content of the liquid medium in which the peptide is initially dissolved.  
30       Preferably, the liquid medium is buffered to about pH 6.5 to about pH 7.5. The buffer preferably includes a salt, such as sodium chloride. For particularly preferred results, the buffer includes 10 mM sodium phosphate and 100 mM

sodium chloride (and is referred to herein as PBS buffer).

Preferably, the peptide is very pure and extremely dry prior to aggregation. Preferably, the peptide is dissolved rapidly (within seconds) in the liquid medium (e.g., PBS buffer) to ensure complete dissolution to a solution (typically, an initial  $10^{-4}$  M solution of the peptide) that is clear to the eye. If the solution is cloudy upon initial dissolution of the peptide, the aggregate formed may be a poor template for A $\beta$  deposition. The solution is then vigorously agitated (e.g., at about 800 revolutions per minute using mechanical stirring). It is envisioned that it would also be possible to aggregate peptide while it is immobilized to a solid support, such as a resin used in the preparation of the peptide, but only if a portion of the peptide is immobilized (i.e., tethered).

Although not necessarily desired, an aggregation enhancing or promoting agent may be combined with the peptide. For example, the enhancing agent may be a small amount of pre-formed aggregate of the peptide, an amount of amyloid plaque derived from Alzheimer's disease tissue, silk, or other substance capable of expediting the aggregation, as for example, a metal ion, or a detergent. If such an agent is used, it is believed that it should be added only after the peptide is completely dissolved to form a clear solution as described above.

Once an aggregate is formed, it can be used directly in an assay, such as a screening assay for agents that inhibit A $\beta$  deposition. If so, the assay will typically require a separation step to separate the aggregate from the undeposited A $\beta$  peptide. Such separation techniques are well known to one of skill in the art and include, for example, centrifugation, filtration, etc.

Preferably, once an aggregate is formed it is immobilized. This typically will make separation of bound and free material easier and faster. Immobilization by a variety of techniques is possible, as discussed above. Preferably, the aggregate is immobilized in a matrix comprising an organic polymer. The organic polymer can be any of a variety of organic polymers. Preferably, they are water soluble and produce a porous polymeric matrix that allows for diffusion of the A $\beta$  tracer into and out of the matrix. Examples of suitable polymers include a wide variety of gelatins (preferably, type B gelatin),

laminins, heparins (preferably, having a molecular weight of about 50,000 to about 150,000), and mixtures thereof. Such polymers are commercially available from a variety of sources, including J.T. Baker, Fisher, and Sigma.

The immobilization method includes the steps of combining an aqueous composition (typically, in the form of a suspension) of aggregated peptide with an aqueous composition (typically, in the form of a solution) of an organic polymer. This step is typically carried out at a temperature effective to dissolve the polymer (on a macroscopic level). In a particularly preferred method, the polymer is initially in a very dry, free flowing state. It is then dissolved in water. For gelatin, a temperature of about 50°C to about 60°C is typically used for dissolution, whereas for laminin and heparin, room temperature is generally sufficient. The aqueous composition of aggregated peptide is then combined with the solution of the organic polymer at the temperature of dissolution of the polymer. Alternatively, the polymer can be dissolved at a higher temperature and cooled before combining it with the aggregate suspension at a temperature that does not destroy the binding capacity of the aggregate. Preferably, the aqueous composition of aggregated peptide is at a pH of about 6.5 to about 7.5 and includes a concentration of aggregated peptide of about 0.1 µg/ml to about 100 µg/ml (these concentrations levels are based on an ideal uniform distribution of the aggregate in the liquid medium).

The resultant mixture (typically, in the form of a suspension) is then dried at a temperature that does not destroy the effectiveness of the aggregate as a template for Aβ deposition. This can occur if either too high a temperature or too low a temperature is used. Preferably, the drying step occurs at a temperature of about 40°C to about 57°C. Prior to drying, the mixture is typically transferred to a desired substrate for use in a screening assay. For example, the mixture is preferably transferred to multi-well plates, although slides or other presentation formats can be used. During transfer it is typically desirable to keep the aggregate in suspension, thereby allowing for uniform concentration of aggregate in the polymeric matrix.

Naturally occurring or synthetic β-amyloid peptide, active fragments thereof, or active modifications of the β-amyloid peptide and its

fragments are preferably used in preparing the synthetic amyloid of the present invention as well as in the A $\beta$  tracers used in the methods of the present invention, wherein "active" is defined below. That is, they are used not only in making the synthetic amyloid but, when they are labelled, they are used in the screening studies, for example.

Naturally occurring  $\beta$ -amyloid peptide (i.e., A $\beta$  peptide) has a sequence of about 40 amino acids. The exact length of the naturally occurring peptide may vary from about 35 to 43 amino acids, depending on the presence of ragged ends. The sequence of the 42-mer peptide is H-  
 10 DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA-OH [SEQ ID NO: \_\_\_\_], and the sequence of the 40-mer peptide is H-  
 DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV-OH [SEQ ID NO: \_\_\_\_]. As used herein, the terms " $\beta$ -amyloid peptide" and "A $\beta$  peptide" include the naturally occurring peptides (having 35-43 amino acids), as well as  
 15 their synthetic analogs. Thus, these terms are not limited to just one particular peptide. Of these  $\beta$ -amyloid peptides, the 40-mer peptide is preferred in both the preparation of the synthetic amyloid and in the A $\beta$  tracer for use in the screening studies. The term "fragment" refers to peptide portions of the A $\beta$  peptide having as few as about 10 amino acids and up to about 35 amino acids, for example.  
 20 The term "modification" refers to A $\beta$  peptide and its fragments having amino acid substitutions, replacements, rearrangements, stereochemical modifications, etc.

As used herein, abbreviations for the amino acids are as listed in Table 1, as shown below. In addition, abbreviations for peptide termini are as  
 25 follows: "H-" means a free amino group, "-OH" means a free carboxyl group, and  
 "-NH<sub>2</sub>" means a carboxyamide. Sequences are numbered from the amino termini with positions indicated by superscripts.

The  $\beta$ -amyloid peptide nomenclature used herein reflects the  
 30 composition of the peptide and the chemical moiety at the C-terminus of the fragment. For example, A $\beta$ (1-40)-OH is the 40-mer (SEQ. ID NO: \_\_\_\_ ) with a free carboxyl group at the C-terminus. A $\beta$ (1-40)-NH<sub>2</sub> is the derivatized 40-mer,

with a carboxamide at the C-terminus. Alternatively, A $\beta$ (1-40)-OH and A $\beta$ (1-40)-NH<sub>2</sub> are referred to herein as  $\beta$ -amyloid peptide<sup>1-40</sup> and  $\beta$ -amyloid peptide<sup>1-40</sup>-NH<sub>2</sub>, respectively. As a further example, the C-terminal-carboxamidated fragment consisting of residues 25-35 is referred to as A $\beta$ (25-35)-NH<sub>2</sub> or

5 alternatively,  $\beta$ -amyloid peptide<sup>25-35</sup>-NH<sub>2</sub>.

Table I. Amino Acid Codes

Single Letter Code	3-letter Code	Amino Acid
A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

An A $\beta$  tracer can be made by combining  $\beta$ -amyloid peptide,

active fragments thereof, or active modifications of  $\beta$ -amyloid peptide and its fragments with an acceptable label by a variety of techniques. The label can be radioactive, antigenic, enzymatic, fluorescent, or any combination thereof. Preferably, the label is radioactive.

5                    Depending on the type of assay used, the peptide used in the synthetic amyloid template may also be labelled. For example, it can include a fluorescent label and be contacted with a fluorescent tracer in a fluorescence quenching assay. Alternatively, the synthetic amyloid template can include a scintillant and be contacted with a radioactive tracer in a scintillation proximity  
10    assay.

                  Among isotopes, any radioactive substance that may be incorporated into the peptide may be used. Preferred isotopes include, but are not limited to,  $^{125}$ Iodine, and  $^{131}$ Iodine; the latter has a shorter half-life and higher energy level. Iodine radioisotopes may be incorporated into the peptide by  
15    oxidative iodination. Also, radioactive iodine may be incorporated by use of Bolton-Hunter reagent to add a 3-iodo-4-hydroxyphenylpropionyl or 3,5-diiodo-4-hydroxypropionyl group to a nucleophile in the peptide.

                  Other isotopes may also be incorporated by reaction with nucleophile groups on peptides. For example, tritium ( $^3$ H) can be incorporated  
20    by reaction with propionyl-N-hydroxysuccinimide, or radioactive sulfur ( $^{35}$ S) can be incorporated by similar reagents. Radioactive phosphorus ( $^{32}$ P) may be incorporated by enzymatic methods. Additionally, various radioactive metal ions, such as  $^{99}$ technetium or  $^{111}$ indium, may be incorporated into  $\beta$ -amyloid peptide or fragments thereof if an appropriate chelating group is added first.

25                    Preferably, the method for obtaining purified labelled peptide (e.g.,  $\beta$ -amyloid peptide, active peptide fragment thereof, or active modification of  $\beta$ -amyloid peptide or fragments thereof) involves using a purified peptide in dry form, i.e., lyophilized. This is then dissolved in a suitable reaction buffer and oxidative radioiodination is carried out to produce a labelled peptide  
30    (preferably monoiodinated), which is then isolated. Preferably, the concentration of the reaction buffer is above about 0.1 M (more preferably, about 0.5 M sodium phosphate) and the pH is about 7-8 (more preferably, about 7.5). It is



preferred that the oxidative radioiodination take place rapidly; more preferably, the time between dissolving the peptide in the reaction buffer and initiation of the isolation step is less than about two minutes, most preferably, less than about one minute. Also, it is preferred that the molar ratio of peptide to radioactive  
5 iodine be greater than about 10:1. For labelled peptides that contain one or more oxidized methionine sidechains, it is particularly advantageous to reduce these sidechains after isolation of the labelled peptide, followed by isolation of the moniodinated, reduced form of the labelled peptide. Preferably, the reduction reaction is about 90 minutes or less.

10 For detection in *in vitro* assays according to the present invention, enzyme labelling is also useful. Among the preferred enzyme labels are peroxidases such as horseradish peroxidase (HRP), or phosphatases such as alkaline phosphatase. Modifying a peptide by adding an antigenic group that will bind with an antibody allows indirect detection of the peptide itself. For  
15 example, the antigen digoxigenin can be linked to an oligonucleotide or peptide, and then visualized with a labelled digoxigenin-specific antibody, or labelled anti-antibody.

Although less sensitive than radioisotopes, fluorophores may also be incorporated into the peptide and detected according to known fluorescent  
20 detection techniques. Examples of suitable fluorophores include fluorescein, rhodamine, Texas Red, and the like. Direct or indirect chemiluminescent labels may also be used according to the invention, such as dioxetanes. For example, the peptide would be modified with a group that is capable of emitting light as it decomposes. In addition, an avidin-biotin system may be used to detect the  
25 peptide or peptide fragment in an *in vitro* assay. For example, a peptide may be functionalized with biotin, and avidin or streptavidin added to detect the peptide.

As used herein, fragments/modifications of  $\beta$ -amyloid peptide include peptides having all or part of the sequence of amino acids found in  $\beta$ -amyloid peptide, and further include derivatizations on the amino- or carboxy-  
30 terminal ends, such as amidation, or at other locations on the peptide. The fragments/modifications may be isolated from those peptides found naturally in the amyloid series, or, alternatively, synthesized in the laboratory or derivatized

in the laboratory, as, for example, with C-terminal amidated peptide fragments. Polyethylene glycol (PEG) may be conjugated to peptides for pharmaceutical use to improve solubility, availability, lifetime, etc. Fragments/modifications further include  $\beta$ -amyloid peptides having substituted amino acids, as for example, a  
5 fragment containing glutamine instead of the wild-type glutamate at position 22 (e.g., Q22-A $\beta$ (1-40)-OH). Alternatively, a fragment may be lacking a native amino acid altogether, which is indicated by the "Des-" prefix in the name (e.g., (des-A2)-A $\beta$ (1-40)-OH, which is a 39-mer lacking the alanine at position 2).

Labelled fragments/modifications can be combined with amyloid  
10 plaque derived from Alzheimer's disease tissue and tested to determine their activity. Activity is evidenced, for example, by the ability of the fragments/modifications to deposit on these materials, as described in Applicants' Assignees' U.S. Patent Application Serial No. 08/304,585 filed September 12, 1994. "Active" fragments/modifications as used herein are  
15 defined as those that show at least about 5% of the deposition activity of A $\beta$ (1-40)-OH (wild type, which is in the all L form) on naturally occurring amyloid plaque. Examples of "active" fragments/modifications of  $\beta$ -amyloid peptide include A $\beta$ (1-40)-NH<sub>2</sub>, N<sup>α</sup>-biotin-A $\beta$ (1-40)-OH, (des-A2)-A $\beta$ (1-40)-OH, A $\beta$ (1-35)-NH<sub>2</sub>, A $\beta$ (10-35)-NH<sub>2</sub>, Q22-A $\beta$ (10-35)-NH<sub>2</sub>, I20-A $\beta$ (10-35)-NH<sub>2</sub>, V21-  
20 A $\beta$ (10-35)-NH<sub>2</sub>, A $\beta$ (10-35)-OH, Q22-A $\beta$ (1-40)-OH, also known as Dutch variant, familial AD. In addition, others that are "active" include A $\beta$ (1-40)-OH and A $\beta$ (1-42)-OH. Fragments/modifications that are not active (i.e., that exhibit less than about 5% of the deposition level of A $\beta$ (1-40)-OH (wild type, which is in the all L form) on naturally occurring amyloid plaque include, for example, rat  
25 A $\beta$ (1-40)-OH, A $\beta$ (1-28)-OH, A $\beta$ (25-35)-NH<sub>2</sub>, A $\beta$ (40-1)-OH, T17,T19-A $\beta$ (10-35)-NH<sub>2</sub>, Y22-A $\beta$ (22-35)-NH<sub>2</sub>, and A $\beta$ (1-40)-OH (all D form).

It is interesting to note that A $\beta$  deposition on synthetic amyloid is stereochemically specific. For example, the all D form of A $\beta$ (1-40)-OH does not bind to a template containing an aggregate of the all L form of A $\beta$ (1-40)-OH;  
30 however, it does bind to a template containing an aggregate of the all D form. Thus, methods of the present invention can be used to establish activity (i.e., binding capability as defined above) of the fragments/modifications relative to

naturally occurring amyloid plaques or synthetic amyloid containing other similar peptides. For example, the synthetic amyloid of the present invention can also be used in determining the activity of fragments/modifications of naturally occurring  $\beta$ -amyloid peptide. Once identified, such

5 fragments/modifications can be used in place of naturally occurring  $\beta$ -amyloid peptides in drug screening assays, and for further study into the basis for the pathology of Alzheimer's disease. For example, a useful fragment is  $\beta$ -amyloid peptide<sup>10-35</sup>-NH<sub>2</sub> [also known as A $\beta$ (10-35)-NH<sub>2</sub>], which displays about 1/3 the activity of A $\beta$ (1-40)-OH, yet is small enough to make possible the determination

10 of the first solution structure (i.e., the three-dimensional structure) of a  $\beta$ -amyloid peptide, solved via the application of nuclear magnetic spectroscopy (NMR). This fragment also shows pH dependence similar to that exhibited by A $\beta$ (1-40)-OH and is folding- and plaque-competent. Thus, this fragment and the deduced solution structure is useful as a reasonable model system for

15 investigating the amyloidogenic structure/activity relationship of the full length peptide, and can serve as a basis for the rational design of drugs to combat Alzheimer's disease and other amyloidosis-producing diseases.

The present invention also provides useful methods to detect, monitor, and screen potential therapeutic agents for affecting Alzheimer's disease

20 or other amyloidosis. In particular, methods for *in vitro* screening of agents that are capable of inhibiting or enhancing A $\beta$  deposition, including the ability to break up and, in certain cases, to inhibit formation or growth of plaques, are provided.

Specifically, an A $\beta$  tracer is combined with a sample of synthetic

25 amyloid and a potential deposition-affecting agent, and the effect on deposition of the A $\beta$  tracer onto the synthetic amyloid is observed. This provides a simpler, more cost-effective drug screen because the synthetic amyloid functions as a model for plaque deposits. Preferably, in a deposition assay, the concentration of A $\beta$  tracer is below about 1 nM, which corresponds to a physiological

30 concentration. Higher concentrations of A $\beta$  tracer can be used, however, if so desired. The synthetic amyloid and A $\beta$  tracer are preferably combined in an acceptable liquid medium having a pH of greater than about pH 4 and less than

about pH 9, and more preferably, about pH 5 to about pH 8, and the assay conducted at a temperature of about 4°C to about 50°C. Incubation typically occurs over a period of about 0.5 hour to about 4 hours. Prior to adding the tracer or agent to be tested, the synthetic amyloid is preferably contacted with a blocking protein, such as BSA or cytochrome-C, for example, in a buffer. For example, a buffer of pH 7.5 having a 0.1% concentration of BSA, can be used. This is desirable to reduce the background noise.

Surprisingly, A $\beta$  tracers bind to synthetic amyloid of the present invention with great affinity, and in a way very similar to their binding to human AD (Alzheimer's disease) amyloid tissue. Among the properties that deposition onto synthetic amyloid and deposition onto authentic human AD plaques share are pH dependence, first order kinetics, structure/activity of the tracers, acceleration or inhibition by the same agents at the same concentrations, and high sensitivity. Deposition onto synthetic amyloid is carried out at an optimal pH of about pH 5 to about pH 8, more preferably, about pH 6.5 to about pH 7.0. The pH optimum for deposition onto AD tissue is preferably between about pH 6.5 and pH 8.5. Significantly, deposition onto AD tissue and onto synthetic amyloid both occur at physiological pH (pH 6-9), and both show a qualitatively similar pH dependence, as shown in Figure 3.

The *in vitro* detecting and monitoring techniques according to the present invention can be qualitative or quantitative. The presence of bound labelled peptide may be detected according to known techniques appropriate for the particular labelling agent and method used (e.g., radioisotope, fluorophore, enzyme, antigen), the particular peptide used (e.g., A $\beta$ (1-40), A $\beta$ (10-35)), and other factors of the assay. In addition, the method of detecting radioactive isotopes will vary according to the isotope and its corresponding energy level. For example, a gamma counter is capable of detecting <sup>125</sup>Iodine, but not tritium (<sup>3</sup>H) or <sup>35</sup>Sulfur.

Where a radioisotope is used to label the peptide, the A $\beta$  tracer/synthetic amyloid complex may be detected by various known radioisotope detection techniques. For example, positron emission tomography may be used to detect isotopes that emit positrons such as radioactive <sup>18</sup>fluorine

or  $^{11}\text{C}$  carbon, gamma counters to detect radioactive  $^{125}\text{I}$  iodine, and scintillation counting methods in the case of tritium ( $^3\text{H}$ ). Nuclear magnetic resonance imaging may also be used, in which case the label would contain a magnetically active particle.

5                   Methods of screening potential deposition-affecting agents using synthetic amyloid of the present invention can be conducted on slides or in standard 96 well plates or other multi-well plates (e.g., 384-well plates), for example. Preferably, the plates or slides are of polyvinyl chloride or glass, and more preferably, polyvinyl chloride. Materials that are known to provide  
10                   inadequate results include polystyrene, polypropylene, and polycarbonate. Using such immobilization techniques advantageously eliminates the need for separation techniques, such as centrifugation.

                  Thus, methods of screening of the present invention can be easily automated and performed by industrial robots. For example, using 20 pM  $^{125}\text{I}$ -  
15                   A $\beta$ (1-40)-OH as tracer, and 1  $\mu\text{g}$  of synthetic amyloid as template, several thousand counts can be deposited in two hours. In contrast, typically 100 pM radiolabelled tracer and 5 mg AD tissue is used in the brain homogenate assay.

                  Thus, a very important and useful aspect of this invention is that it allows drug screening for Alzheimer's disease to be carried out at physiological,  
20                   i.e., sub-nanomolar, concentrations of  $\beta$ -amyloid peptide or peptide fragments. For instance, drugs that affect aggregation behavior at high concentrations of the  $\beta$ -amyloid peptide may be ineffective at physiological concentrations, and the present invention allows for testing under conditions that more nearly represent those found *in vivo*.

25

### Experimental Protocol

**Synthetic Peptides:** A $\beta$  peptides were purchased (QCB, Inc., Hopkinton, MA) or prepared by fluorenyl-methoxycarbonyl chemistry, purified to near homogeneity (>98%) by preparative high performance liquid  
30                   chromatography (HPLC) as described in Applicants' Assignees' U.S. Patent Application Serial No. 08/304,585 filed September 12, 1994 entitled "Labelled  $\beta$ -Amyloid Peptide and Methods for use in Detecting Alzheimer's Disease,"

lyophilized and stored at -30 °C. Peptides were characterized by laser desorption mass spectrometry, amino acid analysis, and reverse-phase HPLC with satisfactory results in all cases. Except where noted, all experiments were performed with A $\beta$ (1-40), the major form of A $\beta$  found in human cerebrospinal fluid, which has the sequence H-DAEFRHDSGYEVHHQKL VFFAEDVGSNKGAIIGLMVGGVV-OH (SEQ ID NO: \_\_\_\_\_). Thus, unless otherwise noted, all synthetic amyloid templates and A $\beta$  tracers for the examples were made using A $\beta$ (1-40)-OH.

**Preparation of Synthetic Amyloid Plates: Fibrillar A $\beta$**

aggregates were prepared from solutions of 10<sup>-4</sup> M A $\beta$  in filtered phosphate buffered saline (PBS, 10 mM sodium phosphate, 100 mM sodium chloride, 7.5 pH), stored and characterized as described in D.T. Weldon et al., Soc. Neurosci. Abs., **22**, 475 (1996). Briefly, A $\beta$ (1-40) was dissolved in PBS rapidly (within 2 seconds) and the resulting solution was initially clear with no evidence of flocculation or incomplete dissolution. To produce radiolabeled A $\beta$ (1-40) aggregates for plate stability assays, <sup>125</sup>I-labeled A $\beta$ (1-40) (see below) was added to unlabeled peptide in PBS to yield a molar ratio of labeled to unlabeled A $\beta$  of 1:5000 and a total A $\beta$  concentration of 10<sup>-4</sup> M. Immediately following dissolution, peptide solutions (7 ml each) were allowed to incubate under vigorous agitation (using mechanical stirring in 15-ml, 17-mm diameter, round-bottomed tubes with a 16 mm TEFLON-coated rounded tip vane magnetic stir bar at approximately 800 rpm or sufficient speed to create a funneling vortex) at approximately 23°C for 24-26 hours. Following incubation, the peptide solutions were distinctly turbid and >80% of the peptide could be sedimented by centrifugation (10 minutes at 15,000 g). The aggregated A $\beta$  suspensions were aliquotted, frozen on dry ice, and stored at -20°C until use. Aggregates from the preparative procedure described above were characterized by Thioflavin S and Congo red staining with positive results in each case, examined for size distribution using fluorescence confocal microscopy as described in D.T. Weldon et al., Soc. Neurosci. Abs., **22**, 475 (1996), and found to have fibrillar morphology (not shown) similar to amyloid from AD plaques by electron microscopy.

To produce synthetic amyloid plates, aggregated A $\beta$  in PBS (see above) was diluted in an aqueous solution of 0.1% gelatin (Type B gelatin, obtained from J.T. Baker, Phillipsburg, NJ, which was dry prior to preparation of the solution) at 57°C to yield 10  $\mu$ g peptide/ml. The A $\beta$ /gelatin suspension was aliquotted to flexible polyvinyl chloride 96-well assay plates (Dynatech Laboratories, Chantilly, VA, Product No. 001-010-2401) at 1.0  $\mu$ g A $\beta$  peptide per well. Rates of A $\beta$  deposition varied linearly with the amount of A $\beta$  immobilized over a range of 0.2 to 20  $\mu$ g/well. Plates were then dried overnight at 57°C. Following this treatment, approximately half of the added A $\beta$  was immobilized in the wells and stable against dissolution in aqueous buffers for at least 36 hours. For staining and autoradiography experiments, A $\beta$ /gelatin suspensions were prepared as above, aliquotted (50  $\mu$ l) onto presubbed microscope slides, and allowed to dry.

Small changes in the protocol resulted in preparations of low activity. As the formation of ordered fibrils is important to production of the template, alteration of parameters such as pH, salt, temperature, and agitation during fibrillization affect template activity. Active synthetic templates for A $\beta$  deposition can be similarly prepared from other substrates such as A $\beta$ (1-42), which has the sequence H-DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA-OH (SEQ ID NO: \_\_\_\_). Identically prepared templates of reverse sequence A $\beta$  [A $\beta$ (40-1)] and scrambled A $\beta$ (1-40), as described in P.E. Fraser et al., Biochemistry, **31**, 10716-10723 (1992), were inactive (not significantly different from no peptide controls), demonstrating specificity. The use of immobilizing polymers other than gelatin (e.g., laminin, heparin, and the like) extends the utility of the assays to a harsher range of experimental conditions (e.g., temperature, solvent). Adaptation of the methods presented here to 384-well or other multi-well plates is straightforward.

**Radioiodination:** A $\beta$  peptides were radioiodinated as described in J.E. Maggio et al., Proc. Natl. Acad. Sci. USA, **89**, 5462-5466 (1992), and in Applicants' Assignees' U.S. Patent Application Serial No. 08/304,585 filed September 12, 1994, entitled "Labelled  $\beta$ -Amyloid Peptide and Methods for use

in Detecting Alzheimer's Disease," and purified to essentially quantitative specific activity (2000 Ci/mmol,  $10^9$  dpm/mg; one  $^{125}\text{I}$  molecule per  $\text{A}\beta$ ). The labeled peptides were stored at  $-20^\circ\text{C}$  in the eluting HPLC mobile phase with 2-mercaptoethanol (0.5%) added to prevent oxidation during storage. Four  
5 distinct synthetic batches of  $^{125}\text{I}$ - $\text{A}\beta$ (1-40) were used in these experiments with indistinguishable results. The radiolabeled  $\text{A}\beta$  peptide ( $^{125}\text{I}$  at tyrosine-10, methionine-35 as the native thioether) used in these experiments was found to accurately track unlabeled  $\text{A}\beta$  in a wide range of experiments.

**Deposition onto The Synthetic Template:** The synthetic  
10 template (in 96-well plates) was preincubated with buffer (50 mM TrisHCl, 0.1% BSA, pH 7.5, except where noted) for 30 minutes. The pre-incubation buffer was removed and radiolabeled  $\text{A}\beta$  (10 pM to 900 pM) in TrisHCl buffer (except where noted) was added to each well in the presence or absence of test compounds. Following incubation (0.5 to 4 hours), the  $\text{A}\beta$  solution was removed  
15 and each well was washed with buffer ( $3 \times 3$  minutes). The wells were capped, separated and the amount of  $^{125}\text{I}$ - $\text{A}\beta$  deposited quantified by  $\gamma$ -counting. Assays were performed at least in triplicate. The amount of  $^{125}\text{I}$ - $\text{A}\beta$  bound to wells prepared with gelatin solutions containing no  $\text{A}\beta$  aggregates (less than 5% of  $^{125}\text{I}$ - $\text{A}\beta$  bound to  $\text{A}\beta$  aggregate/gelatin treated wells) was subtracted as  
20 background. As the assay is not based on optical detection, colored and/or partially soluble compounds can be readily assayed.

**Deposition onto Amyloid in AD Cortex Preparations:** Brain tissue was collected, pathologically characterized, stored, and processed as previously described in W.P. Esler et al., *Biochemistry*, **35**, 749-757 (1996).  $\text{A}\beta$   
25 deposition assays using AD cortical preparations (homogenates and tissue sections) were performed as described in W.P. Esler et al., *Biochemistry*, **35**, 749-757 (1996); P.W. Mantyh et al., *Bull. Clin. Neurosci.*, **56**, 73-85 (1991); J.E. Maggio et al., *Proc. Natl. Acad. Sci. USA*, **89**, 5462-5466 (1992).

30

## Results

**Figure 1:**  $^{125}\text{I}$ - $\text{A}\beta$  deposits readily onto amyloid in AD cortex (a) and onto the synthetic amyloid template of the present invention (c) but not



onto comparable preparations of amyloid-free (control) cortex (b) or preparations of unaggregated A $\beta$  (d). Radiolabeled A $\beta$  (100 pM) was allowed to incubate with slide mounted sections of AD cortex (a), control cortex (b), synthetic amyloid template (c), or comparable preparations of unaggregated A $\beta$  (d) for three hours as described below. Images shown (a-d) are darkfield autoradiograms with light areas indicating sites of radiolabeled A $\beta$  deposition. Scale bar (panels a-d) = 4 mm. Synthetic amyloid template (e), but not comparable preparations of unaggregated A $\beta$  (f), is tinctorially similar to authentic brain amyloid. Immobilized A $\beta$  fibrils (c) and the comparable preparation of unaggregated A $\beta$  (d) were stained with Thioflavin S or Congo red by standard methods. Shown are fluorescent micrographs of Thioflavin S stained synthetic amyloid template (e) and the comparable preparation of unaggregated A $\beta$  (f). Scale bar = 0.6 mm. Similarly, AD cortex, but not control cortex, contains Thioflavin S positive amyloid with sites of *in vitro* A $\beta$  deposition also Thioflavin S positive. Congo red stained A $\beta$  filaments display typical green-gold birefringence characteristic of amyloid (not shown).

**Figure 2:** A $\beta$  deposition onto AD cortex and onto synthetic amyloid template of the present invention follows a linear time course and is nucleation independent. Time course deposition experiments were performed by measuring the deposition of radiolabeled A $\beta$  onto AD cortex homogenates (a) or synthetic amyloid template (c) as described below. A $\beta$  at approximately 600 pM (■), 300 pM (□), 150 pM (▼), 75 pM (▽), 36 pM (●) and 18 pM (○) was allowed to incubate for 0.5 hour to 4 hours at room temperature. Following washing, the amount of radiolabeled A $\beta$  deposited onto the template was quantified by  $\gamma$ -counting. Error bars represent the SEM of six determinations; error bars not visible are smaller than the symbol. Kinetic plots of A $\beta$  deposition rate onto AD cortex (b) or the synthetic amyloid template of the present invention (d) vs. concentration reveal that the kinetics of A $\beta$  deposition follow first order dependence on the concentration of soluble A $\beta$ . A $\beta$  deposition rates were determined from linear regression of time course data. Plots of deposition rate vs. A $\beta$  concentration for AD cortex (b) or the synthetic amyloid template of the present invention (d) show that deposition rate is linearly dependent ( $r^2 =$

0.993 or 0.999, respectively) on concentration of soluble A $\beta$ . Log-log plots of deposition rate vs. A $\beta$  concentration for AD cortex (**b inset**) or the synthetic amyloid template (**d inset**) show slopes of unity ( $1.07 \pm 0.10$  and  $0.99 \pm 0.04$ , respectively), indicating that A $\beta$  deposition in each case is nucleation independent. Error bars represent the standard errors on the slopes as determined by linear regression. Error bars not visible are smaller than the symbols.

**Figure 3:** A $\beta$  deposition onto AD cortex and the synthetic amyloid template of the present invention follow similar pH activity and structure activity profiles. (a) A $\beta$  deposition onto AD cortex (●) or the synthetic amyloid template of the present invention (○) was measured at several pH values. Buffer systems (50 mM buffer, 10 mM MnCl<sub>2</sub>, 0.1% BSA) used were sodium acetate (pH 1-4), succinic acid and ethylenediamine (pH 3-10), and Tris-HCl (pH 6-9). Data for pH dependence of deposition onto tissue taken from W.P. Esler et al., Biochemistry, **35**, 749-757 (1996). Points represent the mean of at least six determinations with a standard error < 5% of the mean. (b) A $\beta$  structure activity is similar for deposition onto AD cortex and the synthetic amyloid template of the present invention. The rates of deposition of several A $\beta$  analogs and A $\beta$ (1-40) (defined as 100%) onto both AD cortex (abscissa) and the synthetic amyloid template of the present invention (ordinate) were determined as described in the text. Peptides tested were A $\beta$ (1-40) (■), A $\beta$ (10-35)-NH<sub>2</sub> (□), A $\beta$ (10-35)-NH<sub>2</sub> L17T, F19T (●), A $\beta$ (10-35)-NH<sub>2</sub> F20I (▼), A $\beta$ (10-35)-NH<sub>2</sub> A21V (▲) and Y<sup>o</sup>-A $\beta$ (23-35) (○). Correlation between the two systems was high ( $r^2 = 0.98$ ) with a fitted slope of  $0.97 \pm 0.08$ .

**Figure 4:** Deposition inhibitors show similar dose response profile for A $\beta$  deposition onto AD cortex or the synthetic amyloid template of the present invention. Congo red (a) or urea (b) show similar dose response profiles using the synthetic amyloid template of the present invention (○) or AD cortex (●). In order to determine the mechanism of action, 1 mM Congo red, 0.3 M urea, or buffer (50 mM TrisHCl, 0.1% BSA, pH 7.5) was allowed to preincubate with the synthetic amyloid template of the present invention (solid bars) for 4 hours (c). After washing, the template was incubated with radiolabeled A $\beta$  for 2 hours. Preincubation with Congo red (filled bars) led to an

increase in inhibition in comparison to buffer preincubation controls (open bars). Comparable preincubation of the dye with radiolabeled A $\beta$  had no effect (not shown). In contrast, preincubation of the synthetic amyloid template with urea (solid bar) was not significantly ( $p>0.25$ ) different from buffer preincubation control (open bar). Error bars represent the SEM of at least six determinations. \*Significantly ( $p<0.001$ ) different from control. Incubation of AD cortex sections with 100 pM radiolabeled A $\beta$  and 3 mM Congo red (d), buffer (50 mM TrisHCl, 0.1% BSA, pH 7.5) (e) or 1 M urea (f) results in inhibition comparable to that observed with AD cortex homogenates or synthetic amyloid template assays (a,b). Shown are darkfield autoradiograms (d-f) produced by parallel exposure, processing and fixing. Light areas represent sites of A $\beta$  deposition. Scale bar = 7 mm. The most intense areas represent A $\beta$  deposited onto vascular amyloid. Optimum film exposure for illustration of both parenchymal and vascular amyloid results in partial saturation of film overlying the latter. Thus, the effects of compounds on A $\beta$  deposition onto plaques (small foci) are more accurately reflected in the autoradiograms than their effect on A $\beta$  deposition onto vascular amyloid.

**Figure 5:** Screens of the synthetic amyloid template, but not traditional aggregation screens, predicts the potency of effectors of A $\beta$  deposition onto AD cortex. A wide variety of structurally diverse compounds (a) was examined for the ability to inhibit or accelerate A $\beta$  deposition onto synthetic amyloid template and authentic brain amyloid in preparations of AD cortex (b). There was a high correlation for EC<sub>50</sub> of compounds on deposition onto synthetic amyloid template and onto AD cortex. Values reported for MTMA, rifampicin, GuHCl, and urea represent pseudo-EC<sub>50</sub> values determined from the maximum effect observed up to the highest concentration tested (0.7 mM, 2 mM, 1 M, and 1 M respectively). Higher concentrations of MTMA, urea and GuHCl were not tested as they may alter the stability the templates. Since PY and ZnCl<sub>2</sub> increase background binding to amyloid free regions of cortex, values for the effect of these compounds on AD amyloid were determined using rates of deposition onto cortical homogenates or densitometry of section autoradiograms rather than single homogenate time points to avoid

“background” binding artifacts. IDOX had no significant effect on either assay at  $10^{-4}$  M under the conditions described. To determine the degree of correlation for all compounds simultaneously by linear regression,  $EC_{50}$  values for inhibitors and accelerators were given positive and negative signs, respectively.

5 Inactive compounds (IDOX) were not included in the correlation calculations. The correlation coefficient was  $r^2 > 0.99$  when all active compounds were included, and  $r^2 = 0.97$  when the two least potent compounds (GuHCl, urea) were omitted from the regression. The solid line shown in (b) is a diagonal (ideal correlation). (c) The potency for inhibition or acceleration of A $\beta$

10 deposition onto AD cortex was compared with the potency of inhibition or acceleration of aggregation estimated from published reports for rifampicin, ZnCl<sub>2</sub>, MTMA and Congo red. No reliable correlation ( $r^2 = 0.18$ ) for potency in deposition and aggregation was observed for these compounds. Points in the upper left quadrant of (c) represent compounds which inhibit A $\beta$  aggregation but

15 accelerate A $\beta$  deposition onto AD tissue *in vitro*. The degree of correlation between  $EC_{50}$  for aggregation and  $EC_{50}$  for deposition onto AD cortex preparations was determined as described in (b).

### Discussion

20                   **Deposition onto Brain Amyloid and Synthetic Amyloid are Biochemically Similar:** Radiolabeled A $\beta$  readily deposited onto amyloid in unfixed AD cortical tissue preparations in a specific and nonsaturable manner. In all cases, visualization of radiolabeled A $\beta$  binding sites by autoradiography (Figure 1a) showed that A $\beta$  was deposited onto both parenchymal and vascular

25 amyloid sites (not shown). While *in vitro* deposition onto brain amyloid in AD cortex is a very favorable process at physiological A $\beta$  concentration (Figure 1a), essentially no deposition was observed onto comparable preparations of amyloid free (age-matched control) brain tissue (Figure 1b); deposition is thus template dependent.

30                   Aggregates of A $\beta$  tinctorially (Figure 1e) and morphologically similar to AD brain amyloid were prepared and immobilized in a polymer matrix in 96-well plates as described above. The resulting synthetic amyloid

preparation was then examined as a template for A $\beta$  deposition. This form of A $\beta$ , like the plaques of AD brain, served efficiently as a template for deposition of radiolabeled A $\beta$  (Figure 1c), while comparable preparations of A $\beta$  not deliberately aggregated (Figure 1f) did not (Figure 1d).

5                Several biochemical properties of the synthetic amyloid template were examined and compared with A $\beta$  deposition onto the amyloid of AD cortex. Deposition onto AD cortex preparations (Figure 2ab) and the synthetic amyloid preparation (Figure 2cd) proceeded linearly with time, with higher rates observed at higher concentrations of A $\beta$ . When the rate of deposition (as  
10                determined from slopes of time course experiments) was plotted vs. A $\beta$  concentration, first order dependence on soluble A $\beta$  concentration was evident for deposition onto both AD cortex and synthetic amyloid template (Figure 2bd), indicating that deposition in each case is independent of A $\beta$  nucleation and consistent with deposition of monomers. Similarly, deposition onto AD cortex  
15                and synthetic amyloid template shared a common pH activity profile (Figure 3a) with a maximum around neutrality, suggesting that the electrostatic interactions between template and peptide important for deposition are similar in each system. In contrast, aggregation of A $\beta$  shows high order kinetics (the rate-limiting step being nucleation) and a pH optimum of approximately 5.

20                To further investigate the synthetic amyloid template model system, the deposition rates of several A $\beta$  analogs were determined and compared with deposition rates onto AD cortex. As evident from Figure 3b, there was a high correlation ( $r^2 = 0.97$ ) between the deposition rates onto tissue and the synthetic amyloid template for several active, inactive and hyperactive  
25                A $\beta$  congeners. These results further underscore the biochemical similarity between deposition onto AD cortex and the synthetic amyloid template and strongly support the hypothesis that within the somewhat heterogeneous deposits of AD brain, the A $\beta$  amyloid is the actual template for further peptide deposition.

30                **Synthetic Amyloid Template Can Be Used To Identify Deposition Inhibitors:** To evaluate the synthetic amyloid template as a high throughput screen for inhibitors of A $\beta$  deposition, several chemically diverse

compounds were examined for their ability to enhance or inhibit A $\beta$  deposition onto the synthetic amyloid template and authentic AD brain amyloid. The azo dye Congo red (Figure 4a) follows essentially indistinguishable dose response profiles for acceleration and inhibition of A $\beta$  deposition onto AD cortex  
5 homogenates or synthetic amyloid. Comparable similarity was observed for inhibition of deposition onto AD tissue and the synthetic amyloid template by the denaturant urea (Figure 4b). In order to probe the mechanism of inhibition for these compounds, Congo red and urea were allowed to preincubate with radiolabeled A $\beta$  or synthetic amyloid template prior to mixing. Preincubation of  
10 either the synthetic amyloid template or AD tissue with 1 mM Congo red led to significantly ( $p < 0.001$ ) increased inhibition of A $\beta$  deposition compared to preincubation with buffer alone, while inhibition by preincubation with urea was not significantly ( $p > 0.25$ ) different from the control (Figure 4c). Conversely, Congo red preincubation with radiolabeled A $\beta$  resulted in no significant ( $p >$   
15  $0.25$ ) increase in inhibition compared to preincubation control (not shown). These results suggest that the effect of an inhibitor may be either on the template (Congo red) or on the solution-phase A $\beta$  (urea). For example, deposition may be inhibited by "coating" the amyloid template (Congo red) as well as by altering the conformation of the A $\beta$  peptide in solution (urea); the methods presented  
20 here provide a means to identify compounds which work by either or both mechanisms. Congo red (Figure 4d) and urea (Figure 4f) also display significant inhibition of deposition onto AD cortex sections relative to parallel control experiments (Figure 4e).

To further test the ability of the synthetic amyloid template to  
25 identify compounds that affect the rate of A $\beta$  deposition onto AD cortex, several compounds were tested as deposition inhibitors or accelerators using both systems. Comparison of the efficacy of several compounds (Figure 5a) for inhibition and/or acceleration of A $\beta$  deposition onto the synthetic amyloid template or AD cortex (Figure 5b) reveals a significant correlation ( $r^2 > 0.97$ )  
30 between the results on the two templates. Pyronine Y (PY), a cationic dye which inhibits A $\beta$  deposition with significantly greater potency than Congo red (CR), represents an entirely novel class of inhibitor discovered using the synthetic

template described here and subsequently shown to be effective on AD tissue. As the assay is not based on optical detection, colored and/or partially soluble compounds can be readily evaluated.

In contrast, comparison between the potency of the compounds for inhibition of A $\beta$  deposition onto AD cortex and potency for inhibition of A $\beta$  aggregation (Figure 5c), reveals no significant correlation ( $r^2 = 0.18$ ). Thus, there is no reliable correlation between the ability of a compound to inhibit A $\beta$  nucleation and its ability to inhibit A $\beta$  deposition, further underlining the distinction between these two processes. It follows that compounds which prevent A $\beta$  nucleation (the rate-limiting but not the only step in aggregation) are not likely per se to be useful in inhibiting A $\beta$  deposition onto an existing amyloid template, although some compounds (e.g., Congo red, Figure 5c) may affect more than one process in A $\beta$  assembly. Thus different screening tools are required to identify inhibitors of A $\beta$  nucleation (initial amyloid formation) and inhibitors of A $\beta$  deposition (amyloid growth). Quantitative morphological observations suggest that the processes are distinct *in vivo* as well as *in vitro*, although their relative importance is difficult to assess.

Since at the time of diagnosis, patients typically have an abundance of brain amyloid which appears capable of supporting further A $\beta$  deposition by an aggregation independent mechanism, A $\beta$  nucleation inhibitors are more likely to be useful prophylactically than therapeutically; conversely, A $\beta$  deposition inhibitors are more likely to be useful in slowing the progression of AD. Thus, the present invention is advantageous because immobilized aggregates of synthetic A $\beta$  serve as a template for A $\beta$  deposition which closely resembles the authentic template in AD brain. Also, the synthetic amyloid template of the present invention forms a convenient assay for identification and optimization of A $\beta$  deposition inhibitors prior to evaluation in rodent or nonhuman primate model systems.

All patents, patent applications, and publications disclosed herein are incorporated by reference in their entirety, as if individually incorporated. The preceding specific embodiments are illustrative of the practice of the invention. It is to be understood, therefore, that other expedients known to those

skilled in the art or disclosed herein, may be employed without departing from the invention.



**WHAT IS CLAIMED IS:**

1. An immobilized synthetic amyloid comprising one or more peptides aggregated to form a peptide aggregate in a fibrillar morphology, wherein the peptides of the peptide aggregate are chosen such that a  $\beta$ -amyloid peptide, an active fragment thereof, or an active modification of  $\beta$ -amyloid peptide or fragment thereof binds to the peptide aggregate.
2. The immobilized synthetic amyloid of claim 1 wherein the synthetic amyloid comprises one or more aggregated peptides immobilized in an organic polymeric matrix.
3. The immobilized synthetic amyloid of claim 2 wherein the organic polymeric matrix comprises a water soluble polymer.
4. The immobilized synthetic amyloid of claim 3 wherein the water soluble polymer comprises gelatin, laminin, heparin, or mixtures thereof.
5. The immobilized synthetic amyloid of claim 1 wherein the synthetic amyloid is immobilized in a multi-well plate.
6. The immobilized synthetic amyloid of claim 1 wherein the one or more aggregated peptides comprises a  $\beta$ -amyloid peptide, an active fragment thereof, or an active modification of  $\beta$ -amyloid peptide or a fragment thereof.
7. The immobilized synthetic amyloid of claim 6 wherein the one or more aggregated peptides comprises  $A\beta(1-40)$ -OH.
8. A method of making immobilized synthetic amyloid, the method comprising:  
aggregating one or more peptides under conditions effective to form a fibrillar peptide aggregate; wherein the

peptides of the peptide aggregate are chosen such that a  $\beta$ -amyloid peptide, an active fragment thereof, or an active modification of  $\beta$ -amyloid peptide or fragment thereof binds to the peptide aggregate; and

combining the fibrillar aggregate with an organic polymer to form an immobilized synthetic amyloid.

9. The method of claim 8 wherein the step of aggregating one or more peptides comprises:

dissolving one or more peptides in an aqueous medium having a pH of about 6.5 to about 7.5 to form an aqueous peptide solution; and

agitating the aqueous peptide solution under conditions effective to form a fibrillar peptide aggregate.

10. An *in vitro* method of screening an agent capable of affecting A $\beta$  deposition, comprising:

combining a sample of synthetic amyloid with an A $\beta$  tracer and a potential deposition-affecting agent to be screened, for a time effective to allow binding of the A $\beta$  tracer to the synthetic amyloid; wherein the synthetic amyloid comprises one or more aggregated peptides and the A $\beta$  tracer comprises a labelled  $\beta$ -amyloid peptide, active fragment thereof, or active modification of  $\beta$ -amyloid peptide or its fragments;

detecting the amount of A $\beta$  tracer bound to the synthetic amyloid; and

assessing the effect of the agent on the amount of A $\beta$  tracer bound to the synthetic amyloid.

11. The method of claim 10 wherein the concentration of A $\beta$  tracer is subnanomolar.

12. The method of claim 10 wherein the aggregated peptide is in a fibrillar morphology.
13. The method of claim 10 wherein the synthetic amyloid is immobilized.
14. The method of claim 13 wherein the synthetic amyloid comprises one or more aggregated peptides immobilized in an organic polymeric matrix.
15. The method of claim 14 wherein the synthetic amyloid comprises aggregated A $\beta$  peptide, active fragments thereof, or active modifications of A $\beta$  peptide or its fragments, immobilized in an organic polymeric matrix.
16. The method of claim 14 wherein the organic polymeric matrix comprises gelatin.
17. The method of claim 10 wherein the synthetic amyloid comprises aggregated A $\beta$  peptide, active fragments thereof, or active modifications of A $\beta$  peptide or its fragments.
18. The method of claim 17 wherein the synthetic amyloid comprises aggregated A $\beta$  peptide.
19. The method of claim 10 wherein the pH of the mixture of the synthetic amyloid and the A $\beta$  tracer is about 5 to about 8.
20. The method of claim 10 wherein the synthetic amyloid is immobilized in a multi-well plate.
21. The method of claim 20 wherein the synthetic amyloid comprises aggregated A $\beta$  peptide, active fragments thereof, or active modifications of A $\beta$  peptide or its fragments, immobilized in an organic polymeric

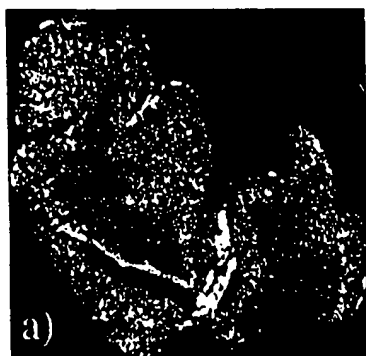
matrix in a multi-well plate.

22. The method of claim 10 wherein the label of the A $\beta$  tracer is a radioactive label, and enzymatic label, fluorescent label, or an antigenic label.
23. The method of claim 22 wherein the label is a radioactive label.
24. The method of claim 23 wherein the labelled peptide is monoradioiodinated and reduced.
25. The method of claim 10 wherein the label of the A $\beta$  tracer is biotin.
26. A multi-well plate comprising synthetic amyloid, wherein the synthetic amyloid comprises one or more peptides aggregated to form a peptide aggregate in a fibrillar morphology immobilized in an organic polymeric matrix, wherein the peptides of the peptide aggregate are chosen such that a  $\beta$ -amyloid peptide, an active fragment thereof, or an active modification of  $\beta$ -amyloid peptide or fragment thereof binds to the peptide aggregate.
27. The multi-well plate of claim 26 wherein the organic polymeric matrix comprises a water soluble polymer.
28. The multi-well plate of claim 27 wherein the one or more aggregated peptides comprises a  $\beta$ -amyloid peptide, an active fragment thereof, or an active modification of  $\beta$ -amyloid peptide or fragment thereof.
29. The multi-well plate of claim 28 wherein the one or more aggregated peptides comprises A $\beta$ (1-40)-OH.
30. A method of identifying an active fragment or modification of  $\beta$ -amyloid

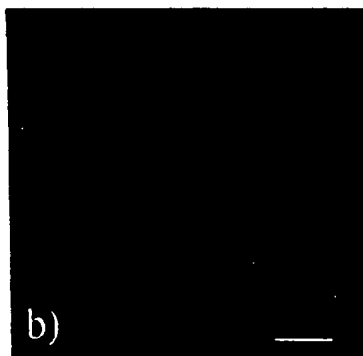
peptide comprising:

- (a) combining a sample of synthetic amyloid with an amount of labelled fragment or modification of  $\beta$ -amyloid peptide for a time effective to allow binding of the labelled fragment or modification to the synthetic amyloid, wherein the synthetic amyloid comprises one or more peptides aggregated to form a peptide aggregate; wherein the peptides of the peptide aggregate are chosen such that a  $\beta$ -amyloid peptide binds to the peptide aggregate; and
- (b) detecting the presence of the labelled peptide fragment or modification bound to the synthetic amyloid.

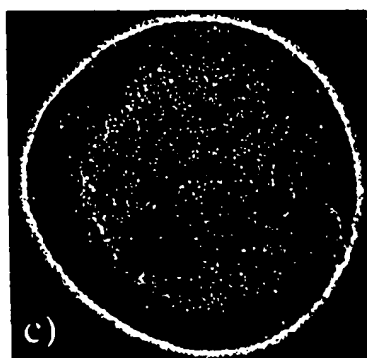
*Fig. 1a*



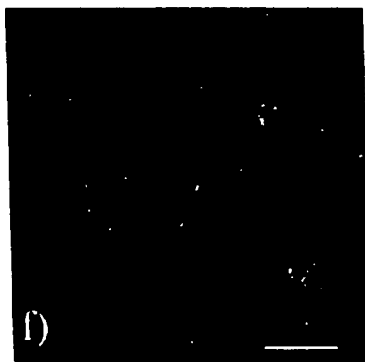
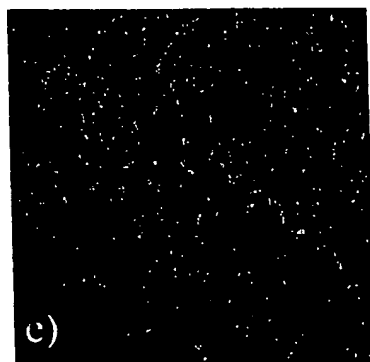
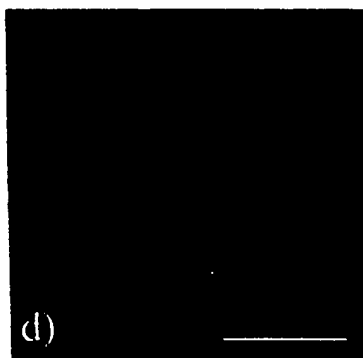
*Fig. 1b*



*Fig. 1c*



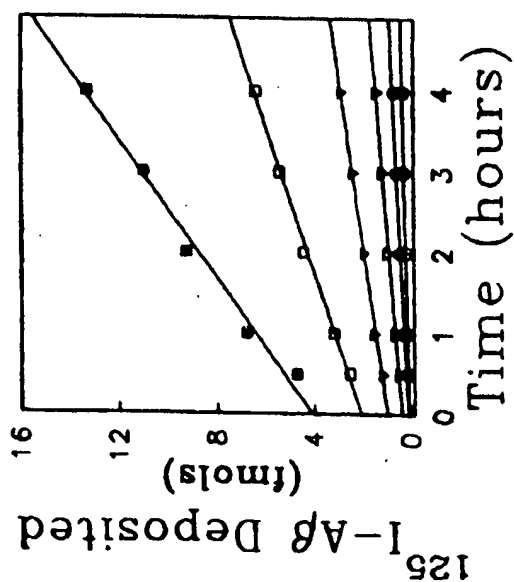
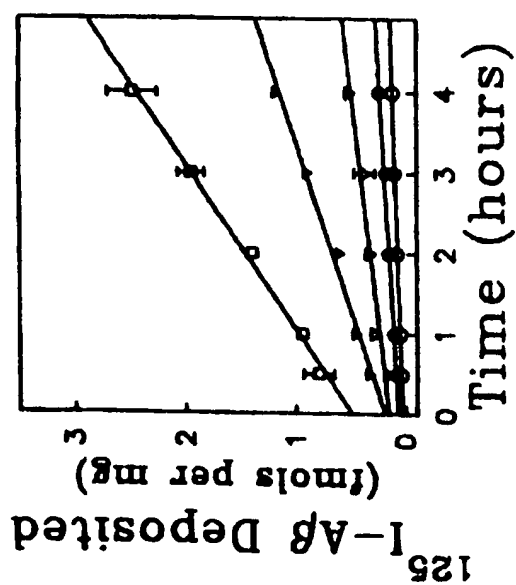
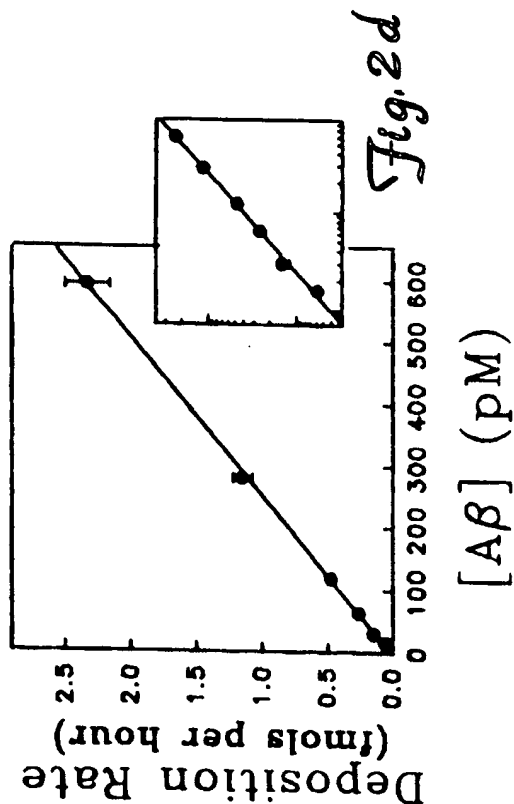
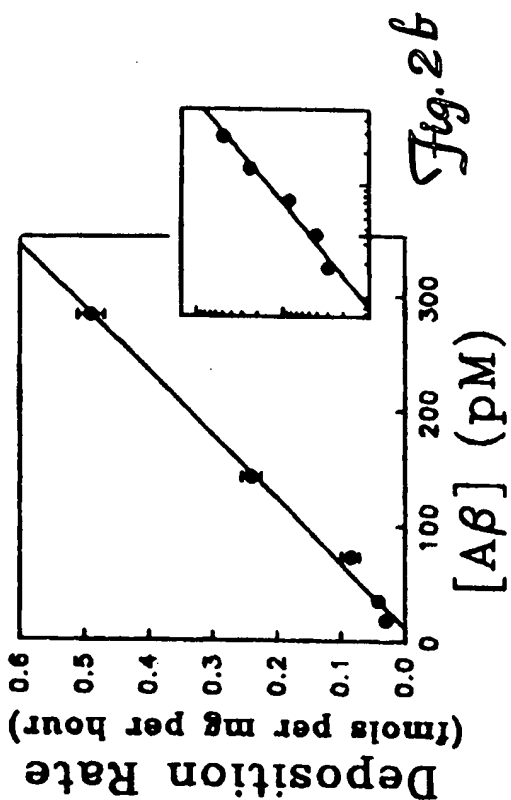
*Fig. 1d*



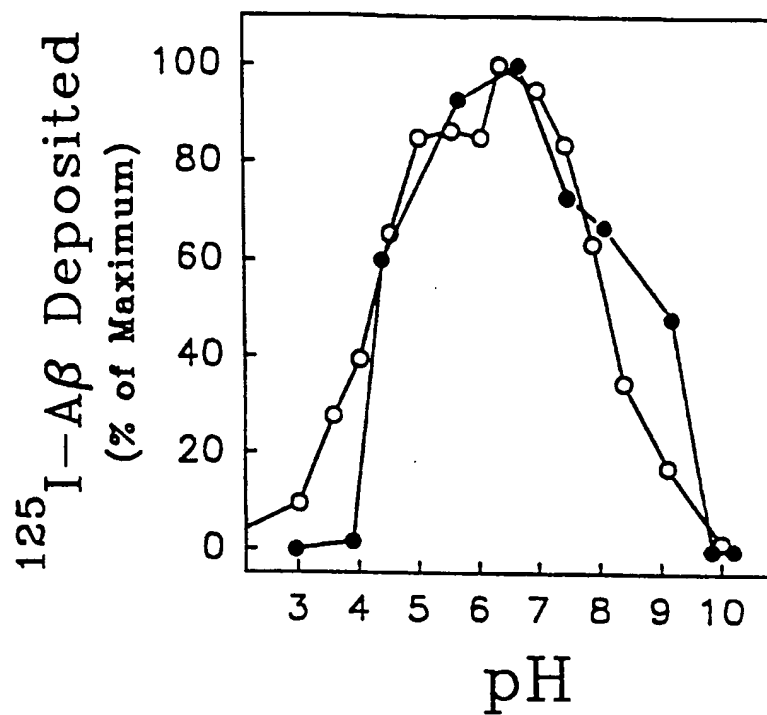
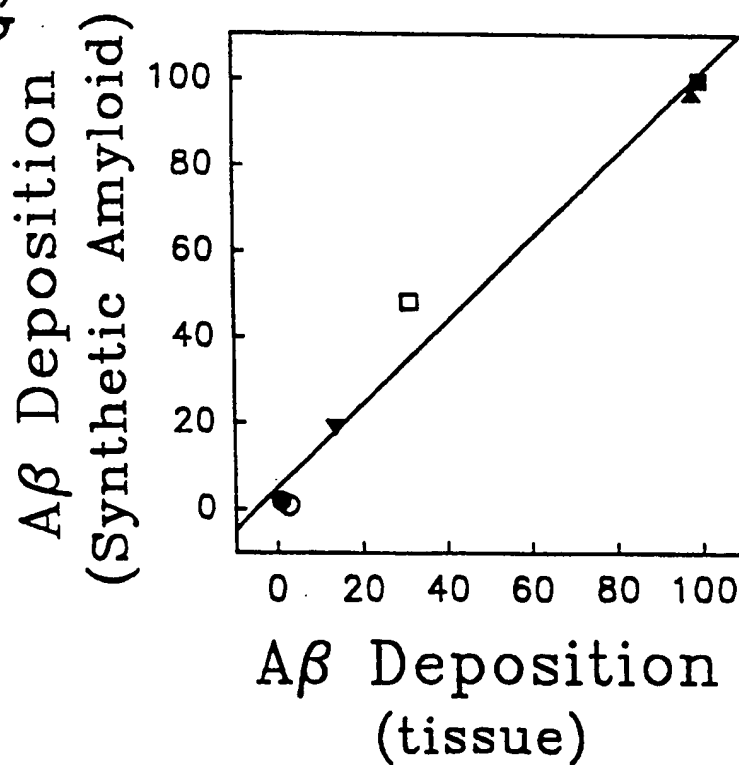
*Fig. 1e*

*Fig. 1f*

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*Fig. 3a**Fig. 3b*



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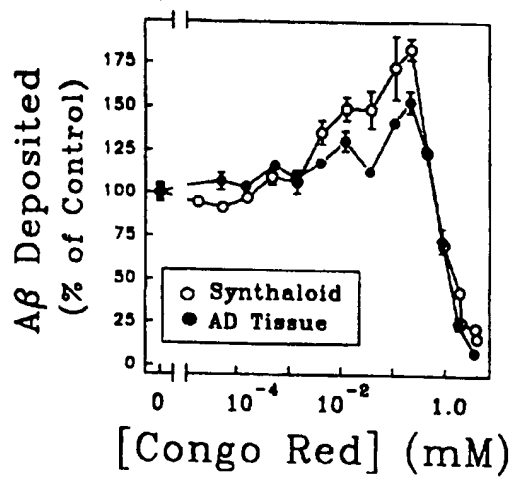


Fig. 4a

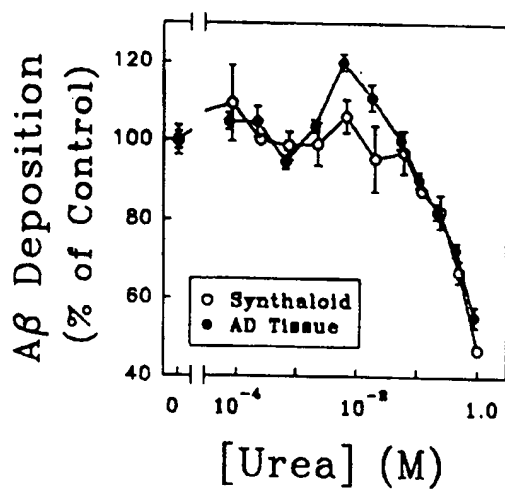


Fig. 4b

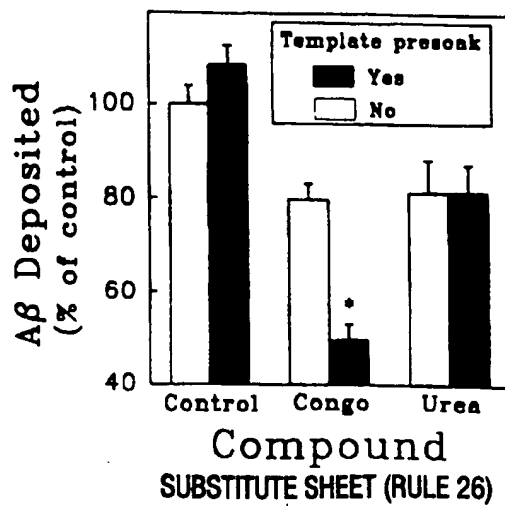
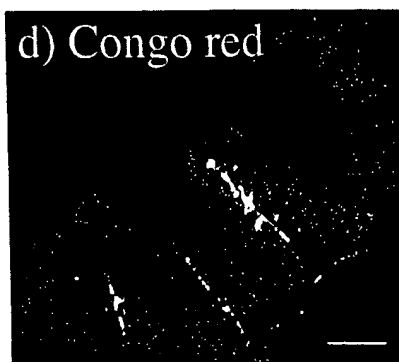
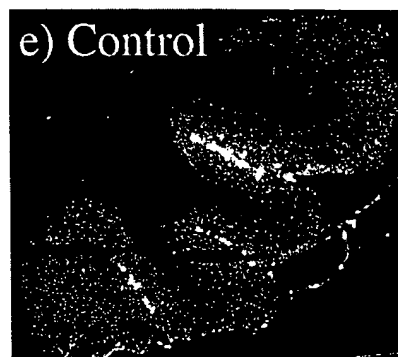


Fig. 4c

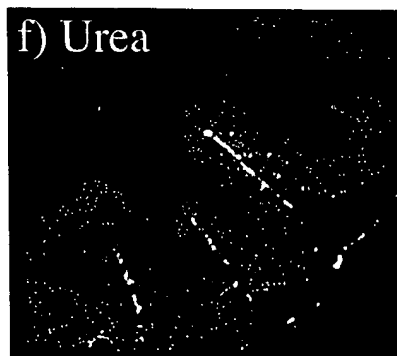
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*Fig. 4 d*

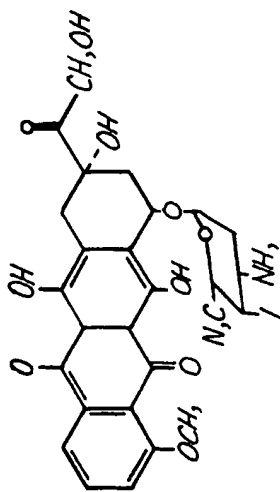
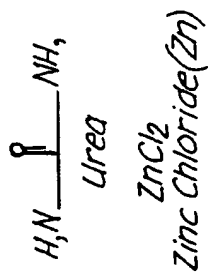
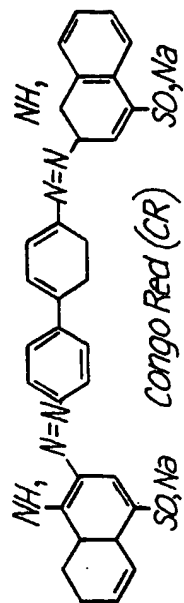


*Fig. 4 e*



*Fig. 4 f*

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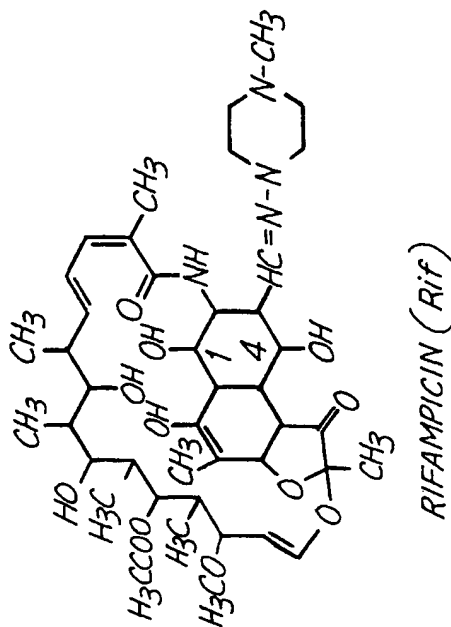
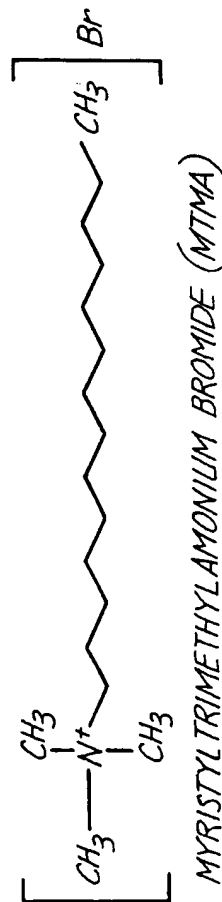
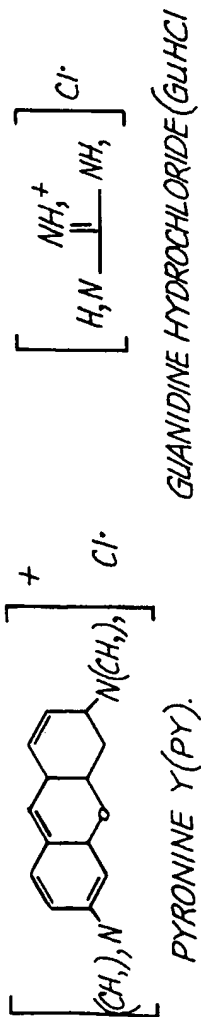


Fig. 5A

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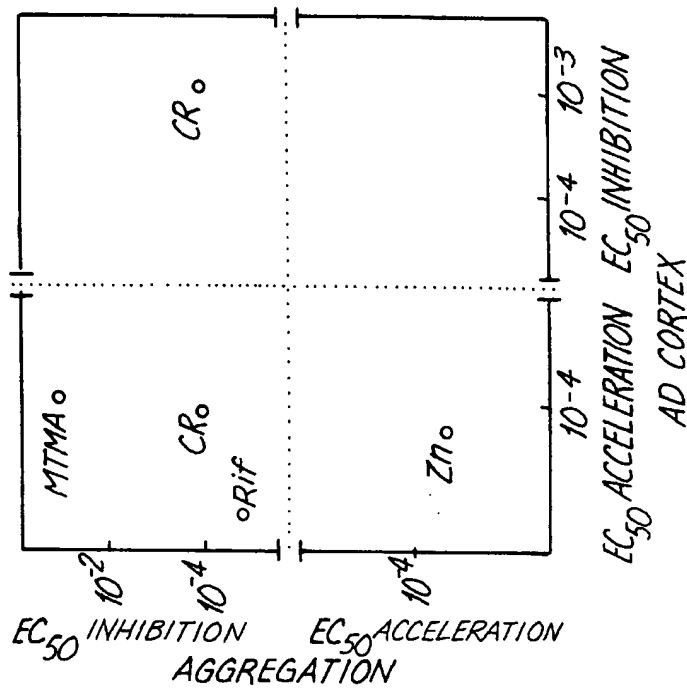


Fig. 5C

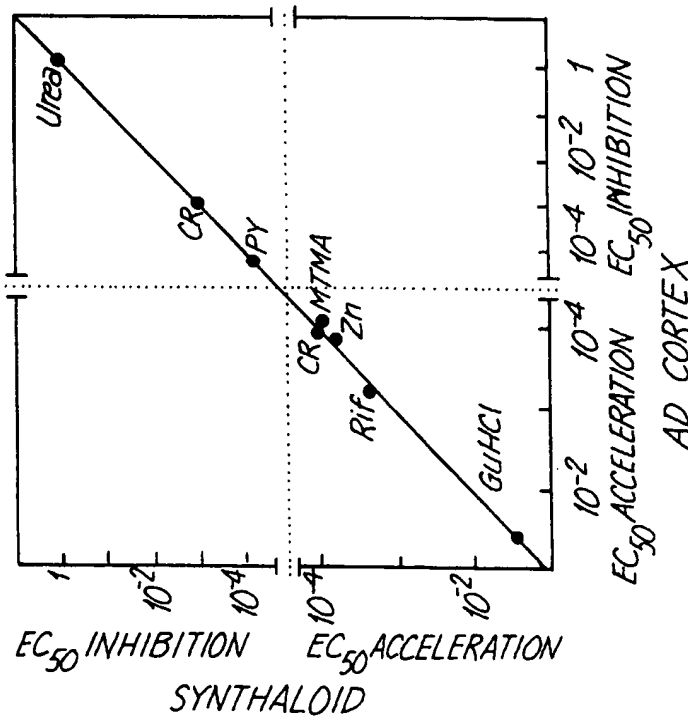


Fig. 5B

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/03136

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 G01N33/68 C07K14/47

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 G01N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 94 09364 A (UNIV DUKE ;STRITTMATTER WARREN J (US)) 28 April 1994 see claims see page 7, line 21 - line 36 ---	1-30
A	HILBICH C ET AL: "AGGREGATION AND SECONDARY STRUCTURE OF SYNTHETIC AMYLOID BETA-A4 PEPTIDES OF ALZHEIMER'S DISEASE." J MOL BIOL 218 (1). 1991. 149-164. CODEN: JMOBAK ISSN: 0022-2836, XP002068206 see page 158, right-hand column - page 159, left-hand column --- -/--	1-30



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

### \* Special categories of cited documents :

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search

16 June 1998

Date of mailing of the international search report

07/07/1998

Name and mailing address of the ISA

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Routledge, B

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 98/03136

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>CHEMICAL ABSTRACTS, vol. 106, no. 13, 30 March 1987 Columbus, Ohio, US; abstract no. 100396, CASTANO, EDUARDO M. ET AL: "In vitro formation of amyloid fibrils from two synthetic peptides of different lengths homologous to Alzheimer's disease.beta.-protein" XP002068207 see abstract &amp; BIOCHEM. BIOPHYS. RES. COMMUN. (1986), 141(2), 782-9 CODEN: BBRC9;ISSN: 0006-291X,</p> <p>-----</p>	1-30

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 98/03136

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9409364 A	28-04-1994	AU 5358494 A	09-05-1994